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DETAILED INSTRUCTIONS FOR THE PERFORMANCE OF THE DISSOLVED OXYGEN AND BIOCHEMICAL OXYGEN DAMAND TESTS

Emery J. Theriault

Public Health Service
Washington, D. C.

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PUBLIC HEALTH SERVICE
HUGH S. CUMMING, *Surgeon General*

DETAILED INSTRUCTIONS FOR THE PERFORMANCE OF THE DISSOLVED OXYGEN AND BIOCHEMICAL OXYGEN DEMAND TESTS

BY

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COMPUTATIONS

Given the initial and final oxygen content of a diluted sample over any period of time, the oxygen demand of the sample for that

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INDUSTRIAL WASTES INVESTIGATIONS

BENCH SHEET—OXYGEN DEMAND AT 20°C.

Source of Sample Cargill Filter No. 1 Sample No. 1202
Nr. Seaway Intake Gates Date 2/21 1920

Bottle Number	Concentration of Sample	Burette Readings c.c. Initial	Burette Readings c.c. Final	Dissolved Oxygen P.P.M. Initial	Dissolved Oxygen P.P.M. Final	Depletion Actual	Depletion Corrected	Oxygen Demand
1	BLANK	14.40 10.00 4.40	14.35 10.00 4.35	8.8	8.7	0.1		
1	1.0	18.75 14.40 4.35	17.55 14.35 3.20	8.7	6.4	2.3	2.2	220
1	2.0	(computed)	19.80 17.55 2.05	8.6	4.1	4.5	4.4	220
5	BLANK	see above	14.25 10.00 4.25	8.8	8.5	0.3		
5	1.0	see above	15.15 14.25 .90	8.7	1.8	6.9	6.6	660

FIGURE 6

period is the corrected difference times a factor for the concentration used. The concentration factor is the reciprocal of the concentration when expressed decimally. Thus for a 5 per cent con-

centration, or, decimally expressed, 0.05, the factor is 20. In th
computation illustrated in Figure 6, it is assumed that 100 ml.
sample were titrated.

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New York, N. Y.

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Health Bull. No. 173.

(3) Moehlman, F. W., Edwards, G. P., and Sworz, G. (1928): Technic and si
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DETAILED INSTRUCTIONS FOR THE PERFORMANCE OF THE DISSOLVED OXYGEN AND BIOCHEMICAL OXYGEN DEMAND TESTS

Foreword

Detailed instructions for the performance of the biochemical oxygen demand test were published in 1918 as Part II of Public Health Bulletin No. 97 ("Studies on the Treatment and Disposal of Industrial Wastes," pp. 78–86), and these instructions were reprinted without change in Public Health Reprint No. 594. Following a survey of sewage treatment plants in 1920, the instructions were revised and were included as Appendix III to Public Health Bulletin No. 132 ("Sewage Treatment in the United States"). With minor additions, based on experience gained in the Illinois River investigations, the directions were repeated in Public Health Bulletin No. 173 ("The Oxygen Demand of Polluted Waters," 1927, Appendix III, pp. 167–173).

Essential precautions in the conduct of the Rideal-Stewart or permanganate modification of the Winkler method were given in Hygienic Laboratory Bulletin No. 96 (1914, pp. 15–36), and detailed instructions for the determination of dissolved oxygen were included in Public Health Bulletin No. 132 (1920, pp. 259–260). These directions were revised and were extended to include a procedure applicable in the presence of iron salts in Public Health Bulletin No. 151 ("The Determination of Dissolved Oxygen by the Winkler Method," 1925).

Numerous requests for information regarding oxygen demand and dissolved oxygen tests have indicated the desirability of assembling this scattered material and of placing it in a more readily usable form.

In the instructions given herewith for the conduct of the oxygen demand test, the only notable departure from previous recommendations is the substitution, under certain conditions, of a buffered synthetic dilution water for stored tap water. This change appears desirable in view of the difficulty experienced in many places in securing a suitable dilution water, owing to the presence of unusual amounts of iron in the tap water supply or to the undesirable pH value, at times, of softened water or of water purified by excess-lime treatment.

As regards the dissolved oxygen test the chief change is the substitution, in the presence of organic matter, of a simplified technique for the permanganate modification of the Winkler method. A method based on preliminary treatment with hypochlorite in alkaline solution is also offered for overcoming the interference due to sulphite-waste liquors.

PART I

DETAILED INSTRUCTIONS FOR THE DETERMINATION OF DISSOLVED OXYGEN BY THE WINKLER METHOD

The Winkler process for the determination of dissolved oxygen (Winkler, 1888) depends on the formation of a precipitate of manganous hydroxide in a glass-stoppered bottle completely filled with the water under examination. The oxygen dissolved in the water is rapidly absorbed by the manganous hydroxide, forming a higher oxide of uncertain composition which, on acidification in the presence of an iodide, releases iodine in quantity stoichiometrically equivalent to the oxygen content of the sample. The liberated iodine is then titrated with a standard solution of sodium thiosulphate in the usual manner. Various modifications of this general procedure are available for counteracting the effect of interfering substances.

REAGENTS

The following solutions are required for the preparation and standardization of the thiosulphate solution:

1. Stock sodium thiosulphate solution.—24.82 grams of $Na_2S_2O_3 \cdot 5H_2O$ per liter, corresponding to a 0.1 M solution. The solution may be preserved by the addition of ammonium carbonate (1.5 grams per liter) and chloroform (5 ml.[1] per liter *after* making up to the mark.) Titrations over long periods of time indicate a rate of deterioration of less than 1 per cent in 6 months with the particular brand of sodium thiosulphate used.

2. Standard sodium thiosulphate solution.—0.025 M. Prepared by diluting 250 ml. of stock thiosulphate solution (0.1 M) to 1 liter or by weighing out 6.205 grams of $Na_2S_2O_3 \cdot 5H_2O$ and making up to 1 liter with distilled water.

The keeping qualities of the 0.025 M thiosulphate solution are somewhat improved by the addition of 5 ml. of chloroform after making up to the mark. This solution, however, will deteriorate quite rapidly and should be made up fresh every few weeks. The strength of the solution may be checked with a standard solution of biniodate.

3. Potassium biniodate solutions.—A stock solution of strength equivalent to that of the 0.1 M thiosulphate contains 3.250 grams of $KIO_3 \cdot HIO_3$ per liter in accordance with the following reaction:

$$2KIO_3 \cdot HIO_3 + 20\ KI + 11\ H_2SO_4 = 11\ K_2SO_4 + 12\ H_2O + 12\ I_2$$

[1] The abbreviation "ml." for milliliter or milliliters is used throughout instead of "c.c."

A biniodate solution equivalent to the 0.025 M thiosulphate solution contains 0.8124 grams of $KIO_3 \cdot HIO_3$ per liter and may be prepared by diluting 250 ml. of the stock biniodate solution to 1 liter.

Potassium biniodate (acid iodate) can readily be obtained sufficiently pure for direct weighing without further purification, and its keeping qualities both in the dry state and in solution leave little to be desired. The titration is exactly the same as when potassium permanganate or bichromate is used to titrate thiosulphate solutions.

Apart from their uses for standardization, these biniodate solutions are also convenient for back titrations, in case of overruns, both in the regular procedure and in the hypochlorite modification.

4. Starch solution.—About 0.5 per cent. Grind 5 or 3 grams of arrowroot starch in a mortar with a small quantity of water. Pour into 1 liter of boiling water, stir, and allow to settle overnight. Use the clear supernatant.

5. Dilute acid for standardization.—3.5 N H_2SO_4 approximately. (One volume of concentrated sulphuric acid poured into 9 volumes of water.)

6. Potassium or sodium iodide crystals.—Free from iodates.

The following reagents are required when the unmodified Winkler procedure is to be used:

7. Manganous sulphate solution.—480 grams of $MnSO_4 \cdot 4H_2O$ or 400 grams of $MnSO_4 \cdot 2H_2O$ dissolved in distilled water, filtered, and made up to 1 liter. When uncertainty exists regarding the water of crystallization, a solution of equivalent strength may be obtained by adjusting the specific gravity of the solution to a value of 1.270 at 20° C. The manganous sulphate solution should liberate only traces of iodine when added to an acidified solution of potassium iodide. (Absence of manganic and ferric salts.)

8. Alkaline-iodide reagent.—500 grams of NaOH (or 700 grams of KOH) and 135 grams of NaI (or 150 grams of KI) per liter. Potassium and sodium salts may be used interchangeably. The reagent should be free from carbonates, as $MnCO_3$ does not react with dissolved oxygen. It should not give any color with starch when diluted and acidified. (Absence of nitrites, iodates, and ferric salts.)

A sodium hydroxide solution free from carbonate is conveniently obtained by siphoning the supernatant from a stock solution of concentrated sodium hydroxide and adjusting the clear solution to a strength of 15 M (corresponding to a specific gravity of about 1.453).

To prepare 1 liter of alkaline-iodide reagent, dissolve 135 grams of NaI in 120 ml. of distilled water and add 835 ml. of 15 M NaOH solution. The reagent obtained conforms very closely to specifications.

9. Concentrated sulphuric acid.—Specific gravity, 1.83–1.84. The strength of this acid is about 18 M (36 N). Hence 1 ml. is equivalent to about 3 ml. of the alkaline-iodide solution.

In addition to the above reagents, the following solutions should be prepared when the Rideal-Stewart modification of the original Winkler method is to be used:

10. Potassium permanganate solution.—6.32 grams of $KMnO_4$ per liter, corresponding to a 0.04 M solution (0.2 N).

11. Oxalate solution.—2 grams of potassium oxalate $(COOK)_2 \cdot H_2O$, in 100 ml. of distilled water, corresponding roughly to a 0.11 M solution (0.22 N). One ml. of this oxalate solution will suffice for the neutralization of about 1.1 ml. of the permanganate solution. Under certain conditions this solution deteriorates rapidly. An oxalic acid solution of equivalent strength (1.4 grams of $(COOH)_2 \cdot 2H_2O$ in 100 ml. of distilled water) may be used.

In the presence of appreciable amounts of iron salts, the following solutions will be required:

12. Fluoride solution.—40 grams of $KF \cdot 2H_2O$ dissolved in distilled water and made up to 100 ml.

13. Phosphoric acid.—85 per cent H_3PO_4.

The following solutions are required when the hypochlorite modification is used:

14. Alkaline-hypochlorite solution.—1 M NaOCl and 0.1 M NaOH. This solution may be prepared by passing chlorine gas through a 0.1 M NaOH solution until the desired amount of NaOCl has been formed.

The pharmaceutical preparation "Hyclorite," if fresh, is approximately molar in respect to NaOCl. On adding 1 volume of 1 M NaCH to 9 volumes of "Hyclorite," a precipitate of $Ca(OH)_2$ is formed which settles out readily. The supernatant liquor is in reasonable conformity with the above specifications.

The alkaline-hypochlorite solution should be kept tightly stoppered when not in use.

15. Iodide solution.—1 M KI or NaI. Prepared by dissolving 17 grams of KI or 15 grams of NaI in distilled water and making up to 100 ml. This solution should be preserved by the addition of 1 ml. of 1 M NaOH to each 100 ml. of solution.

16. Sodium sulphite solution.—0.05 M (0.1 N). Contains 6.3 grams Na_2SO_3 per liter. The keeping properties of this solution are improved by the addition of 1 ml. of 3.5 N H_2SO_4 to each liter of solution.

APPARATUS

1. Sample bottles.—In general, bottles of the type commonly used in oxygen demand determinations (240 to 380 ml.) are very satisfactory. At the start of an investigation it may nevertheless be desirable, for convenience and uniformity, to select a bottle of the largest size (300 ml. capacity) recommended for oxygen demand work in "Standard Methods for the Examination of Water and Sewage" of the American Public Health Association.

In field work, where weight is a factor, reliable results can still be obtained when bottles of smaller capacity are used (say, 125 ml., or even less), the volume of reagents added being correspondingly reduced.

2. Sampling devices.—See Figures 1 and 2.

3. Pipettes.—Terminating in tubes of small bore for delivering reagents near the bottom of the bottles. See Figure 5, Part II.

4. Dosing stand.—For convenience in adding reagents.

SAMPLING FOR DISSOLVED OXYGEN

In general, the sampling arrangements should be such that the liquid in the sampling bottle is displaced several times without entrainment of air bubbles.

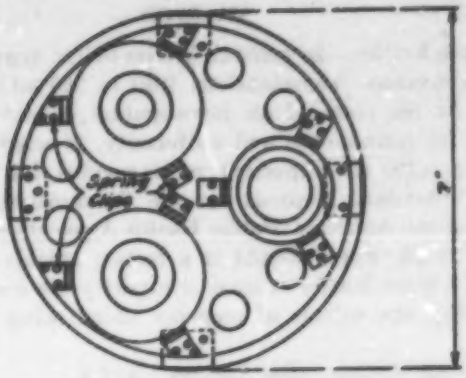
In sampling a water supply a rubber tube extending to the bottom of the bottle may be connected to a tap and the water allowed to overflow for a few minutes.

In sampling from streams, ponds, or tanks of moderate depth, it may be convenient to use the apparatus shown in Figure 1. This apparatus is designed for the simultaneous collection of three samples which may be used, respectively, for bacteriological, oxygen demand, and dissolved oxygen determinations.

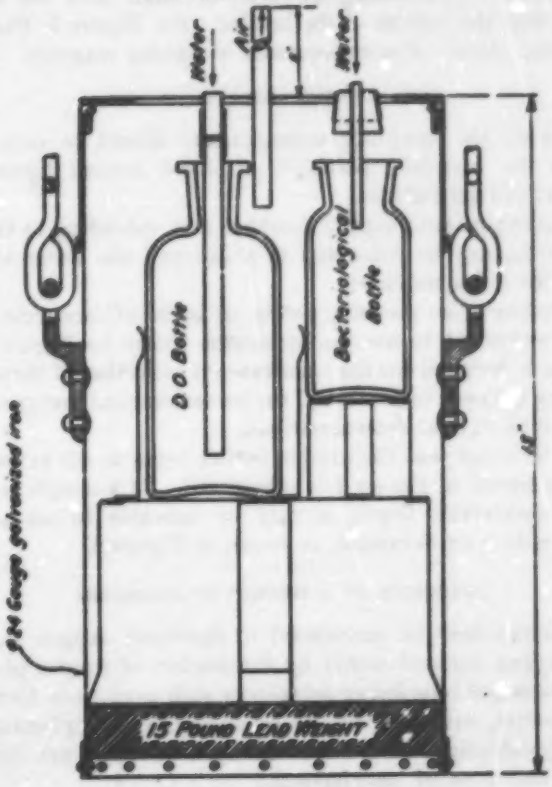
It will be noted that the sample bottles begin to fill as soon as the apparatus shown in Figure 1 is submerged. If a sample is required from a considerable depth, it may be desirable to use a sampler provided with a valve release, as shown in Figure 2.

SELECTION OF A METHOD OF PROCEDURE

Huge errors may be introduced in dissolved oxygen work (and also in oxygen demand work) by the neglect of proper precautions in the presence of interfering substances such as nitrites, ferrous salts, organic matter, sulphites, etc., or by an improper application of the various modifications of the Winkler method which are designed to overcome this type of interference.



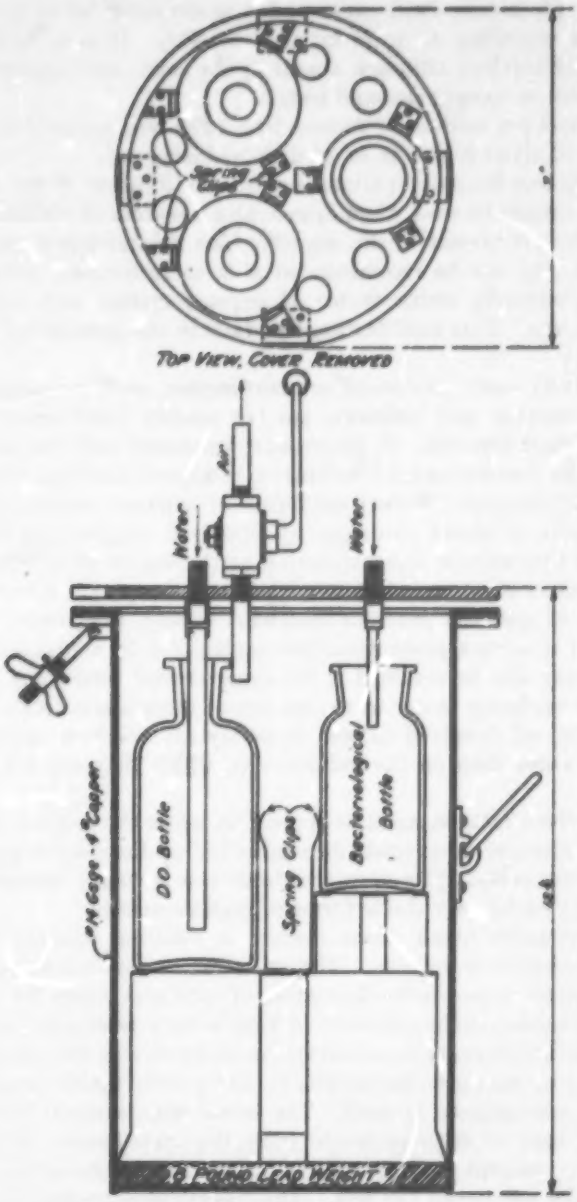
TOP VIEW, COVER REMOVED



SECTIONAL VIEW

SAMPLE COLLECTOR - TYPE A

FIGURE 1



SAMPLE COLLECTOR - TYPE B

FIGURE 2

24226—81——2

As a rule nitrites will cause undue interference when present in amounts exceeding 0.1 p. p. m. of N as NO_2. It is to be borne in mind that nitrites, although absent at the start, may appear during incubation in oxygen demand tests.

One part per million of ferrous iron (Fe) will occasion an apparent loss of about 0.14 p. p. m. of dissolved oxygen.

The Rideal-Stewart (permanganate) modification of the Winkler method should be used when appreciable amounts of nitrites or ferrous salts are present in the sample. The permanganate treatment, however, can not be recommended as a corrective for interference due to relatively stable forms of organic matter such as sugars, starches, etc. This modification also fails in the presence of sulphite wastes.

Relatively stable forms of organic matter, such as sugars (dextrose), starches, and cellulose, are not readily acted upon by permanganate in the cold. A preliminary treatment with permanganate of samples containing such substances is unprofitable and may be actually detrimental. Numerous forms of organic matter, however, are capable of direct oxidation by dissolved oxygen at pH values of 12 or thereabouts (corresponding to the degree of alkalinity obtained when alkaline-iodide is added to a sample). Although the reaction in question proceeds somewhat slowly, low results may be obtained if certain precautions are neglected. In acid solution low results may also be obtained if the experimental conditions are such that the oxidizing power of the manganic compounds formed by the absorption of dissolved oxygen is partly expended on organic materials rather than on the iodides with which they are designed to react.

With these facts in mind, it is possible, using the original Winkler method, to make an accurate determination of dissolved oxygen even though the sample (for instance, fresh raw sewage) contains large amounts of relatively stable forms of organic matter.

Polythionates break down readily in alkaline solution to form sulphites and thiosulphates. The oxidation of such compounds must, therefore, be carried to the formation of sulphates before the addition of the alkaline-iodide solution. A preliminary treatment, according to the Rideal-Stewart modification, is ineffective as the oxidation to sulphates of sulphites, thiosulphates, and polythionates is incomplete when permanganate is used. The error with samples containing 0.25 per cent of digester waste from the manufacture of sulphite pulp may amount to 7 or 8 parts per million of dissolved oxygen, the results appearing too low. The "alkaline-hypochlorite" modification should be used with such wastes.

The directions given below for the determination of dissolved oxygen in the presence of organic matter and sulphite waste are based on unpublished data obtained in critical studies of the Winkler method. A report on these studies is in preparation.

A. The permanganate (Rideal-Stewart) modification

PROCEDURE IN THE PRESENCE OF NITRITES, FERROUS SALTS, ETC.

The detailed procedure given below is based on the Rideal-Stewart or permanganate modification of the Winkler method, and on the fluoride modification presented in Public Health Bulletin No. 151. (For a brief outline see p. 15.)

To avoid too great a dilution of the sample with the reagents, it is desirable that the capacity of the sample bottles should be in the neighborhood of 300 ml. The indicated amounts of reagents, however, may be used even though the capacity of the bottles varies from 240 to 380 ml.

1. Add 0.7 ml. of concentrated sulfuric acid.—It is important that the amount called for should not be exceeded. The 0.7 ml. portion, for that reason, is best added with a 2-ml. pipette.

The acid is allowed to run down the neck of the bottle. All other reagents, however, should be added well below the surface of the liquid.

2. Add enough of permanganate solution to obtain a violet tinge which persists after shaking.

With samples whose behavior is fairly well known, the proper amount of permanganate may be added all at once.

With samples of unknown or variable composition, trial amounts (1 ml. or less) should be added with shaking and a suitable delay allowed between additions. When an additional amount of permanganate appears necessary, it should be added while a faint violet tinge still persists (that is, before the appearance of brownish colorations due to intermediate oxides).

When large amounts of permanganate are required (5 ml. or more) a stronger solution of $KMnO_4$ should be used.

An attempt should be made to keep the amount of permanganate added down to a minimum and the probable excess of permanganate in the solution should be noted.

3. It is customary to allow the samples to stand for 20 minutes after obtaining a violet color which appears to be permanent. This period of standing appears to be based on results obtained in titrations of nitrites with permanganate solutions and it probably refers to the time which must be allowed for the complete oxidation of the nitrites.

In practice, a period of standing of five minutes at ordinary temperatures appears sufficient to reduce the nitrite content to a value which will not cause undue interference.

The oxidation by permanganate solutions of ferrous salts and of sulfides, both mineral and organic, is practically instantaneous. A period of standing of one-half minute should suffice in such cases.

4. Potassium oxalate solution is added to destroy the excess of permanganate whenever the oxidation of the interfering substances (readily oxidizable materials) is judged complete.

As a trial amount which should not be greatly exceeded, it is recommended that 0.5 ml. of the 2 per cent oxalate solution be first added. If after standing for five minutes the color of permanganate still persists, an additional 0.5 ml. portion should be added. When the directions regarding the addition of permanganate have been followed, this second portion should induce complete decolorization.

When a large excess of oxalate is added, the period of decolorization is lengthened and the results obtained are too low.

It is important that perfect decolorization be secured, as even a trace of brownish coloration will react with iodides on acidification, giving results which may be entirely too high. Apparent increases in dissolved oxygen content during incubation may frequently be traced to this source of error.

In the presence of iron salts, decolorization is delayed to a surprising extent (24 hours under certain conditions), even when the correct amount of oxalate has been added. In order to hasten the decolorization when iron salts are known to be present, potassium fluoride (2 ml. of 40 per cent solution) should be added along with the permanganate or at any other stage of the preliminary treatment.

For the reason that ferric salts in the presence of oxalates are readily reduced to the ferrous condition by light (very rapidly so in direct sunlight), the decolorization should proceed in the dark when iron salts are present in appreciable amounts.

5. When the permanganate is fully decolorized, add 1 ml. of manganous sulphate solution followed by 3 ml. of alkaline-iodide reagent. Do not add less than the indicated amounts.

It will be noted that approximately 2 ml. of alkaline-iodide reagent are required for the neutralization of the 0.7 ml. portion of concentrated sulphuric acid added at the start, leaving 1 ml. for combination with the manganous sulphate. The amount of iodide added, however, is three times greater than when 1 ml. each of the two reagents is used. The extra amount of iodide is beneficial in repressing the deleterious effect of the oxalate and, presumably, of other forms of organic matter as well.

ERRATUM

The first lines of the subtitles under sections B and C, pages 11 and 12, respectively, were inadvertently interchanged. The first line of subtitle under section B, page 11, should read, "Procedure in the presence of sulphite-waste liquor (sulphites"; while the first line of subtitle under section C, page 12, should read, "Procedure applicable to pure water and also to waters."

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The foregoing steps constitute the preliminary treatment of a sample for the destruction of nitrites, sulphides, etc., and for the oxidation of ferrous salts to the ferric condition. The subsequent treatment is the same as when the original Winkler method is used, starting with the shaking of the sample after the addition of the Wrinkler reagents. (See sec. 6, p. 12.)

B. Alkaline-hypochlorite modification

PROCEDURE APPLICABLE TO PURE WATERS AND ALSO TO LIQUORS HEAVILY THIOSULPHATES, POLYTHIONATES, ETC.)

For reasons already given, the permanganate modification is ineffective in counteracting the interference of sulphite wastes. With such samples a practical degree of accuracy may be secured through preliminary treatment with hypochlorite and iodine, as described below. At best, however, this procedure gives low results, the error with 0.25 per cent of digester waste amounting to about 1 part per million. Delays at any stage of the process should be avoided.

A preliminary treatment with hypochlorite is effective in counteracting interference due to nitrites or ferrous salts although, for this purpose, it is perhaps simpler to use the permanganate treatment.

1. Add enough of the alkaline-hypochlorite reagent (1 M NaOCl and 0.1 M NaOH) to oxidise the sample, avoiding a great excess. With river water polluted with sulphite waste, 0.2 ml. of the alkaline-hypochlorite reagent may be added as a trial amount. In oxygen demand work, add 1 ml. of hypochlorite solution for each ml. of digester waste present in the bottle under examination.

2. Mix well by inverting rapidly a few times. The duration of this treatment should not greatly exceed 20 seconds.

If the period of contact with the alkaline-hypochlorite is unduly prolonged, low results will be obtained, owing to the absorption of dissolved oxygen by the decomposition products of the organic matter.

3. Acidify the sample by adding 1 ml. of 3.5 N H_2SO_4 (10 per cent solution) and add 1 ml. of 1 M KI (or NaI) to release iodine. Shake.

4. Neutralize the liberated iodine with 0.1 N sodium sulphite, using 0.2 ml. of starch solution as an inside indicator. If the end point is overstepped, the blue color should be restored with 0.1 ml. portions of 0.1 N potassium biniodate.

The reaction between sulphites and iodine to form sulphates is complete only in very dilute solution. Under the given experimental conditions the results will be relatively low when more than 3 ml. of 0.1 N sulphite are required for the neutralization of the iodine.

On the other hand, if only 0.1 ml. or thereabouts of 0.1 *N* sulphite are required, it may be assumed that an insufficient amount of alkaline-hypochlorite has been used.

5. Add 1 ml. of manganous sulphate solution and 1.8 ml. of alkaline-iodide solution.

A slight excess of the alkaline-iodide reagent is used in order to neutralize the acid added during the preliminary treatment.

For the subsequent treatment see section 6 below, under the regular Winkler procedure.

C. The original Winkler method

PROCEDURE IN THE PRESENCE OF SULPHITE-WASTE LIQUOR (SULPHITES, POLLUTED WITH RELATIVELY STABLE FORMS OF ORGANIC MATTER IN THE ABSENCE OF READILY OXIDIZABLE MINERAL OR ORGANIC SUBSTANCES

In the directions given below, the volume of reagents added (2 ml. each of manganous sulphate solution, alkaline-iodide solution, and concentrated sulphuric acid) is based on the assumption that large sample bottles (capacity of 360 to 380 ml.) are being used. In the presence of organic matter the same amounts of reagents also may profitably be added even when the capacity of the sample bottles is in the neighborhood of 300 ml.

With bottles of the size recommended in "Standard Methods"[1] (240 to 270 ml. for the dissolved oxygen determination and 250 to 300 ml. for the oxygen demand tests) the volume of reagents may be reduced to 1 ml.

With smaller bottles (125 ml. or less) it will be sufficient to add 0.5 ml. each of the reagents.

It is assumed that ferrous salts and other readily oxidizable substances are not present in more than traces. Otherwise a preliminary treatment (steps 1 to 4 of the modified procedures) should be applied.

5. Add 2 ml. each of the manganous sulphate and alkaline-iodide solutions. For the sake of minimizing the loss of dissolved oxygen by oxidation of organic materials at high pH values, the manganous sulphate solution should be added first. These reagents should be added well below the surface of the liquid so as to avoid loss of precipitate on replacing the stopper.

6. Shake well, by inverting the bottle several times, so as to distribute the precipitate uniformly throughout the bottle.

In the absence of large amounts of organic matter, the period of standing in the presence of alkaline-iodide is unimportant. Under this condition, it is permissible to pass from one bottle to another of a series of parallel determinations, allowing each precipitate to

[1] 1925 edition, pp. 59 and 78.

settle until it is contained in the lower half of the bottle. As the precipitate settles readily, the dissolved oxygen in the upper part of a bottle will not be completely absorbed unless the period of shaking is suitably prolonged. If uncertainty exists on this point, or else if a clear supernatant is not obtained, the shaking should be repeated. This precaution should especially be borne in mind when reduced amounts of reagents are used.

In the presence of large amounts of organic matter, low results will be obtained if the period of standing in the presence of alkaline-iodide is unduly prolonged. For this reason the test should be carried through on individual samples when interference by organic matter is suspected and the period of alkalinization should be reduced to the minimum time consistent with the complete absorption of dissolved oxygen. For reasons already given, it can not be assumed that organic materials are absent when the permanganate treatment has been used.

When 2 ml. of manganous sulphate have been added, it may safely be assumed that the absorption of dissolved oxygen will be practically complete when a precipitate of manganous hydroxide is agitated *continuously* in a closed bottle for 20 to 25 seconds. When 1 ml. only of manganous sulphate is used, as in the modified procedures, a longer period of shaking (40 to 50 seconds) is desirable. If the sample is then acidified as soon as the precipitate has settled, accurate results will be obtained in the presence of organic matter in amounts corresponding to 1,000 parts per million of either dextrose or peptone.

Larger amounts of organic materials may be present (say 5,000 p. p. m. or 0.5 per cent of dextrose) without introducing an appreciable error, if the sample is acidified before the precipitate has settled (that is, immediately after shaking), suitable allowance being made for the slight loss of precipitate by displacement on adding the acid. This procedure should be followed with sulphite waste.

7. *Acidify with 2 ml. of concentrated sulphuric acid*, allowing the acid to run down the neck of the bottle. A weaker solution (50 per cent H_2SO_4) may be used.

A lesser amount (1 ml.) of sulphuric acid may be used. The amount recommended (2 ml.) is advantageous, however, in repressing the deleterious effect of oxalates and other forms of organic matter.

The use of hydrochloric acid is contraindicated in the presence of iron salts.

8. *To avoid the reduction of manganic salts* by organic materials, it is desirable that the sample be shaken as soon as possible after the addition of acid when organic substances are present.

The liberated iodine diffuses slowly. It should be uniformly distributed throughout the bottle before withdrawing an aliquot part of the sample for titration.

9. In the absence of ferric salts and certain forms of organic matter (acetic acid, for example), it may be possible to delay the titration for several days without introducing an appreciable error.

The titration may be delayed for one hour, even in the presence of 100 p. p. m. of ferric iron, if potassium fluoride has been added in the preliminary treatment. In the absence of fluoride, the titration should be performed *immediately* after the final acidification when the ferric iron content exceeds, say, 10 p. p. m.

The titration may be delayed for several days, even in the presence of 100 p. p. m. of iron, if phosphoric acid (4 ml. of 85 per cent H_3PO_4) is substituted for sulphuric acid in the final acidification.

10. A correction for the loss of dissolved oxygen by displacement with the reagents should be applied to the amount measured out for titration. Thus, when 2 ml. each of the manganous sulphate and alkaline-iodide reagents are added to a 300-ml. bottle, the amount measured out for titration should be

$$200 \times \frac{300}{300-4} = 202.7 \text{ ml.}$$

if it is desired to titrate 200 ml. of the original sample. No correction need be applied for the sulphuric acid unless the addition of this reagent has occasioned a loss of precipitate.

When the preliminary treatment with permanganate has been used, a correction should be applied for the following volumes of added reagents:

0.7 ml. of concentrated H_2SO_4.
1.0 ml. of 0.2 *N* $KMnO_4$.
2.0 ml. of alkaline-iodide solution.
1.0 ml. of manganous sulphate solution.
5.7 ml.

When 300-ml. bottles are used, the amount measured out for titration should be

$$200 \times \frac{300}{300-5.7} = 203.8 \text{ ml.}$$

if it is desired to titrate 200 ml. of the original sample.

The assumptions made in deriving this correction factor are only partly correct, as some of the reagents undoubtedly contain dissolved oxygen. No correction has been made for the addition of the oxalate solution, as it is presumably saturated with dissolved oxygen. A correction of this magnitude about balances the tendency of the oxalate to give low results.

For many purposes the titration of 100 ml. of sample will give sufficiently accurate results. Conversely, greater accuracy may be secured by conducting the titration in the bottle itself, after removing 20 ml. of the iodine solution with a pipette.

11. *Titrate with 0.025 M thiosulphate* until the amount of iodine remaining is equivalent to about 0.5 ml. of thiosulphate solution, corresponding to a pale straw color in the sample.

12. *Add 1 or 2 ml. of freshly prepared starch solution* and titrate rapidly to the first disappearance of the blue color. Subsequent recolorations owing to the presence of traces of iron salts or to the catalytic effect of nitrites, etc., should be disregarded.

13. *When several samples are being run at the same time*, four or five aliquot portions may be poured out at one time if the titrations are not delayed, provided that iron salts and nitrites are not present in more than traces.

The loss of iodine by diffusion into the air is appreciable if the oxygen content exceeds, say, 7 p. p. m. and the titration is unduly delayed. Interference by otherwise negligible amounts of nitrites may also be encountered if the sample is exposed to air for several minutes.

14. *When the volume titrated* is equivalent to 200 ml. of the original sample, the dissolved oxygen content in parts per million is numerically the same as the required volume of 0.025 M thiosulphate expressed in milliliters. In general,

$$\text{p. p. m. of dissolved oxygen} = \frac{200\ (\text{ml. of 0.025 M thiosulphate})}{(\text{ml. of sample titrated})}$$

D. Schematic outline for dissolved oxygen determination

For convenience of reference, the following outline has been prepared by condensing the detailed instructions given under "A," "B," and "C."

PRELIMINARY TREATMENT WITH PERMANGANATE AND FLUORIDE

1. Add 0.7 ml. of concentrated sulphuric acid.

2. Add enough of permanganate solution to obtain a violet tinge. Add 2 ml. of fluoride solution if appreciable amounts of iron salts are present.

3. Allow to stand until the reaction of the permanganate with readily oxidizable substances is judged complete (5 minutes or less).

4. Decolorize with the minimum possible amount of oxalate solution. The decolorization should proceed in the dark when iron salts are present.

PRELIMINARY TREATMENT WITH ALKALINE-HYPOCHLORITE

1. Add a suitable amount of alkaline-hypochlorite reagent.

2. Shake for 20 seconds.

3. Add 1 ml. each of 3.5 N H_2SO_4 (10 per cent solution) and 1 M KI. Shake.

4. Add 0.2 ml. of starch solution and neutralize the iodine with 0.05 M (0.1 N) sulphite solution, restoring the blue color with 0.1 ml. portions of 0.1 N biniodate.

THE WINKLER PROCEDURE

5. When a preliminary treatment with permanganate has been applied, add 1 ml. of manganous sulphate and 3 ml. of alkaline-iodide reagent.

When the alkaline-hypochlorite modification has been used, add 1 ml. of manganous sulphate and 1.3 ml. of alkaline-iodide.

When a preliminary treatment is unnecessary the test is started by adding 2 ml. each of the manganous sulphate and alkaline-iodide solutions. Lesser amounts may be added under certain conditions.

6. In the practical absence of organic matter it is permissible to shake each bottle vigorously for a few seconds, passing from one bottle to another of a series. A second shaking is advisable. In the presence of much organic matter treat each sample individually as directed in detailed instructions.

7. Acidify with concentrated sulphuric acid (1 or 2 ml.) depending on the conditions.

8. Distribute the liberated iodine uniformly throughout the bottle.

THE TITRATION

9. In general, the titrations should be commenced within one hour after the final acidification. (See detailed instructions for tolerance in special cases.)

10. For the titration it is convenient to pour out a portion of the iodine solution which corresponds to 100 or 200 ml. of the original sample.

11. Titrate to a pale straw color with 0.025 M thiosulphate.

12. Add 1 or 2 ml. of starch solution and titrate rapidly to the first disappearance of the blue color, neglecting subsequent recolorations.

13. The titration should not be delayed, once the sample has been poured out.

COMPUTATIONS

14. In general,

$$\text{p. p. m. of dissolved oxygen} = \frac{200\ (\text{ml. of } 0.025\ M \text{ thiosulphate})}{(\text{ml. of sample titrated})}$$

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PART II

DETAILED INSTRUCTIONS FOR THE DETERMINATION OF BIOCHEMICAL OXYGEN DEMAND BY THE EXCESS-OXYGEN (DILUTION) METHOD

The determination of the biochemical oxygen demand of a sample by the excess-oxygen (dilution) method consists, essentially, in measuring the decrease in dissolved oxygen which occurs when a liquid containing organic matter and capable of supporting biological growth is incubated under standard conditions. As water at ordinary temperatures will hold only about 9 p. p. m. of dissolved oxygen in equilibrium with air, it is necessary to dilute the samples so as to secure an excess of dissolved oxygen whenever the oxygen demand exceeds, say 7 or 8 p. p. m. Various other complications are introduced when the samples must be seeded, neutralized, etc., prior to incubation.

The following method of procedure has been used extensively in stream pollution and similar investigations conducted by the United States Public Health Service. With suitable working arrangements the test can be carried out rapidly and conveniently. Close adherence to analytical details is necessary, however, if consistent results are to be expected.

APPARATUS

Sampling devices: See Part I, Figures 1 and 2.

Incubation bottles: Low form, provided with accurately fitting glass stoppers. A 300-ml. bottle of the type pictured in Figure 3 is convenient.

Carboys: 20-liter capacity (5 gallons) to store water for dilution purposes.

Seals: See Figure 4.

Siphons and pipettes: See Figure 5.

Incubator: Capable of regulation within one degree of 20° C.

SAMPLING

Methods and apparatus for the collection of samples from streams, ponds, etc., have been considered in Part I under the heading "Sampling for dissolved oxygen."

Methods of sampling for sewage and industrial wastes are largely determined by local facilities.

In the ideal case, hourly samples proportioned to the flow of waste at the time of sampling (for example, 10 ml. per million gallons, if the average flow is 10 million gallons) are collected over a full 24-hour period and composited for analysis. The availability of direct-reading, flow-measuring devices is implied.

FIGURE 5

In the absence of flow-measuring devices, hourly samples of, say, 150 ml., may be collected throughout the day for compositing.

In rare cases where the character of the waste or sewage varies little from day to day and where its composition throughout the day has been fully established, it may be sufficient to collect a single sample at a particular hour which corresponds to average conditions. In general, however, no reliance can be placed on catch samples.

Wide-mouthed bottles, provided with wire handles, are used for sampling sewage, and sample collectors should be instructed to dip well below the surface of the liquid. As soon as possible after collection, a suitable amount of the hourly samples is transferred to a one-gallon bottle kept on ice or in an ice-box. The change in the oxygen demand of an iced sample within 24 hours is small.

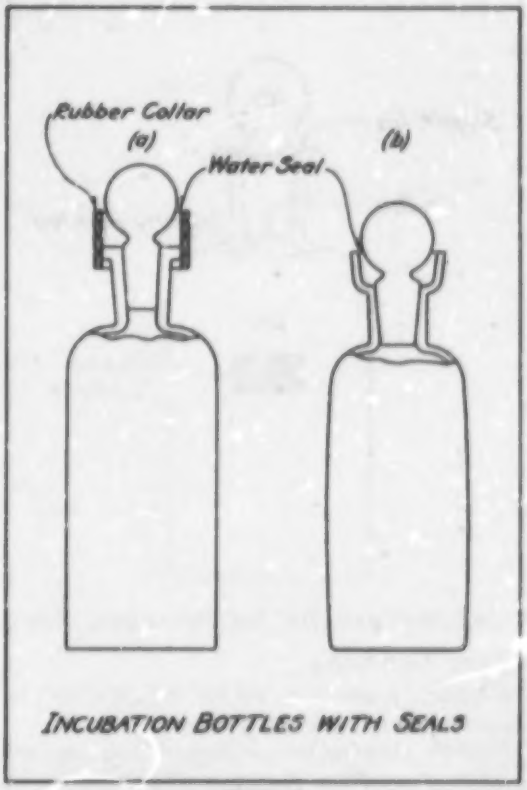

FIGURE 4

There is a need for the further development of automatic devices for the sampling of sewage and industrial wastes.

In collecting samples for oxygen demand tests, information should also be secured regarding the temperature of the samples and the rate of flow. In the absence of such information, the interpretation of the data may be difficult or impossible.

THE DILUTION WATER

It is essential that the dilution water should possess the following characteristics at the time of use:

(*a*) Its 10-day oxygen demand should be less than 0.6 p. p. m., or its 5-day demand should be less than 0.4 p. p. m.

(*b*) It should neither be supersaturated nor greatly undersaturated with dissolved oxygen at the standard temperature of 20° C.;

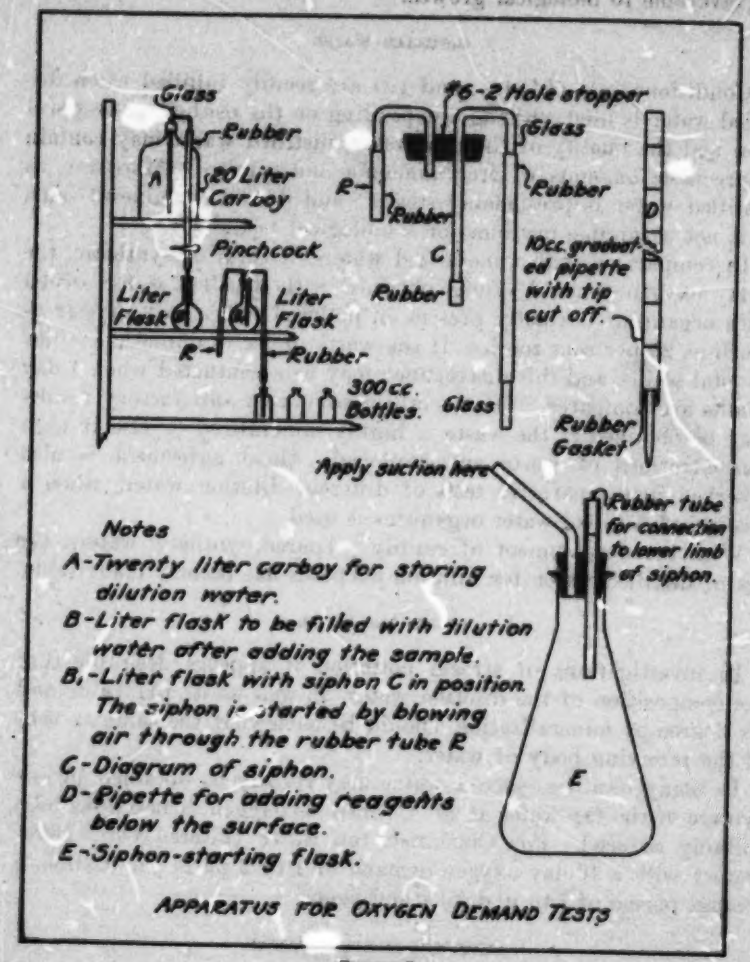

FIGURE 5

that is, it should contain between 3.0 and 9.0 p. p. m. of dissolved oxygen.

(*c*) The temperature of the dilution water should be approximately 20° C.

(*d*) The water should be free from interfering substances, such as iron salts and nitrites, and from inhibitory substances, such as free chlorine, chloramines, and copper salts.

(*e*) The pH value and the mineral salt content of the water should be favorable to biological growth.

1. DISTILLED WATER

Conditions (*a*), (*b*), (*c*), and (*d*) are readily fulfilled when distilled water is used, although, depending on the method of preparation and the quality of the raw water, distilled water may contain appreciable amounts of free ammonia and nitrites. Moreover, as distilled water is presumably sterile [1] and devoid of mineral salts, it is not a suitable medium for a biological test.

In comparison with mineralized waters, natural or synthetic, the 5-day oxygen demand results obtained with distilled water seeded with organisms normally present in polluted water may appear as much as 25 per cent too low if the waste under examination is low in total solids, and this discrepancy may be accentuated when 1-day results are compared. On the other hand, very satisfactory results may be obtained if the waste is highly mineralized or else if high concentrations of waste are employed. Good agreement is also observed in comparative tests of different dilution waters when a seeding of distilled water organisms is used.

With the development of readily prepared synthetic waters, the use of distilled water for dilution purposes has become inadvisable.

2. STORED TAP WATER

In investigations of stream pollution it appears desirable that the composition of the dilution water, as well as its pH value and its degree of mineralization, should be somewhat the same as that of the receiving body of water.

In many localities such a water may readily be obtained by the storage of the tap water at 20° C. until its oxygen demand has been suitably reduced. For Cincinnati tap water (filtered Ohio River water) with a 10-day oxygen demand of 1 or 2 parts per million, a storage period of 7 to 10 days is sufficient.

3. SYNTHETIC DILUTION WATERS

It is recognized that very frequently it may be impossible to secure a suitable dilution water from natural sources of supply. Moreover, for purposes of comparison it may be desirable to provide a dilution water of readily reproducible characteristics.

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[1] Under ordinary conditions of storage, distilled water may contain a few varieties of bacteria. Plankton, however, are generally absent.

Extended comparisons of various synthetic dilution waters[2] have indicated that, within the usual limits of error, a considerable latitude is allowable in the degree of mineralization and in the nature of the mineral salts, provided that the pH value and, especially, the seeding of the dilution waters, is somewhat the same. There is a need, however, for a repetition of this work with different sewages and with various types of industrial wastes before drawing definite conclusions regarding the suitability of a given synthetic water. The recommendations which follow are therefore to be regarded as tentative.

A. PHOSPHATE SOLUTIONS

As a standard of reference, it appears advisable to use a synthetic water buffered at a pH value which corresponds to average conditions (say, pH 7.2).

A buffer solution of this description is readily prepared by dissolving 34 grams of potassium acid phosphate (KH_2PO_4) in about 500 ml. of distilled water. A solution of sodium hydroxide (40 grams of stick NaOH per liter, corresponding to 1 M NaOH) is then added until a pH value of 7.2 is reached. About 175 ml. of 1 M NaOH will be required. The mixture is then diluted to one liter.

The adjustment of the pH value of the phosphate solution is not critical and it may be accomplished with color standards or charts, or else, with sufficient accuracy, by removing portions of 1 ml. and testing with brom thymol blue until a color change is observed.

The dilution water is then prepared by adding 20 ml. of the buffer solution to 16 liters of distilled water (free from copper salts, etc.). One liter of the stock buffer solution will therefore suffice for the preparation of 800 liters of dilution water.

Excluding the mineral salts added along with the sample, the salt content of this water will be about 50 p. p. m., corresponding to a relatively soft water. If the actual salt content appears to be of consequence, the amount of stock phosphate solution added might be doubled in localities where natural waters of average hardness are found and the figure might be quadrupled when hard waters are encountered.

In using a dilution water of this description it is tacitly assumed that necessary mineral nutrients, such as calcium, sulphur, iron, etc., will be supplied by the water. This assumption should be generally true with moderate concentrations of sewage and industrial wastes.

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[2] A report on these studies is in preparation.

In special cases it may be desirable to furnish a more varied mineral content by introducing the following reagents:

40 ml. of 0.1 M $CaCl_2$ solution.
40 ml. of 0.04 M $MgSo_4$ solution.
8 ml. of 0.001 M $FeCl_3$ solution.

These reagents are added with stirring to 16 liters of the phosphate dilution water described above. The 0.1 M $CaCl_2$ solution contains 18.3 grams of $CaCl_2 \cdot 4H_2O$ per liter; the 0.04 M $MgSO_4$ solution contains 9.9 grams of $MgSO_4 \cdot 7H_2O$ per liter, and the 0.001 M $FeCl_3$ solution contains 0.27 gram of $FeCl_3 \cdot 6H_2O$ per liter.

It will be noted that, for the purposes of the test, the hydrion concentration of a phosphate dilution water may be regarded as constant within a wide range of dilution. It is also possible, without greatly disturbing the mineral salt content, to shift from the arbitrarily selected value of pH 7.2 to the particular pH value within the ordinary range of bacterial growth which appears best suited to local conditions. These properties of the phosphate solutions make them especially valuable as standards of reference.

B. BICARBONATE SOLUTION

The use of a dilute solution of sodium bicarbonate (0.3 gram of $NaHCO_3$ per liter, corresponding to 300 p. p. m.) for dilution purposes commends itself for simplicity of preparation. Unfortunately the hydrion concentration (pH 8.0, or thereabouts) of such a solution is relatively low, and it is not capable of ready adjustment to meet local conditions as in the case of the phosphate solutions. Moreover, it is to be borne in mind that, owing to the gradual loss of carbon dioxide, the hydroxyl ion concentration of a bicarbonate solution may reach undesirable values. Factors to be considered in this connection are the purity of the bicarbonate itself (absence of carbonate) and the conditions of preparation and storage of the bicarbonate solution.

On the basis of these theoretical objections, it does not appear advisable to recommend the use of the readily prepared bicarbonate solution for dilution purposes until more data from different laboratories have been accumulated regarding the effect of pH on the rate and extent of deoxygenation in natural waters. Extended series of observations in this laboratory have indicated that with Cincinnati sewage (Third Street sewer) the 5-day oxygen demand results are not greatly affected when a bicarbonate solution containing 300 p. p. m. of $NaHCO_3$ is used instead of a phosphate solution buffered at pH 7.2. Over shorter periods of incubation the phosphate dilution water appears to give somewhat higher results (absence of lag). Conversely, when the periods of incubation are prolonged beyond

five days, the bicarbonate water gives the highest result owing, possibly, to the favorable effect of relatively high pH values on the activity of nitrifying organisms.

PRELIMINARY TREATMENT OF THE SAMPLE

1. ADJUSTMENT OF THE PH VALUE

Biological activity may be inhibited if a sample is unduly acid (pH 5.5 or less) or if it is unduly alkaline (pH 8.5 or greater). In such cases the adjustment of the hydrion concentration to a standard value (say, pH 7.2) or else to a value corresponding to that of the receiving body of water (as a rule, pH 6.5 to 8.0) appears to be the most logical procedure.

If the sample is unduly acid, a dilute solution of Na_2CO_3 (10.6 grams of Na_2CO_3 per liter gives a 0.2 M solution) is added until a test portion reacts definitely alkaline to brom thymol blue.

If the sample is unduly alkaline, a dilute solution of HCl (17.1 ml of concentrated HCl, sp. gr. 1.19, per liter, gives a 0.2 M solution) is added until the sample reacts acid to cresol red.

It is not advisable to add an arbitrary amount of acid or alkali, once the desired pH value has been reached.

It is to be borne in mind that when acid or alkaline samples are highly diluted, the buffer strength of the dilution water may be sufficient to maintain a favorable pH value without adjustment.

2. SEEDING

As the test depends on the symbiotic activity of bacteria and plankton, it is necessary to insure at all times the presence of both classes of organisms. This precaution is of particular importance in the examination of industrial wastes which have been boiled or which possess a high degree of acidity or causticity. The same remarks apply to chlorinated raw sewage or sewage effluents. Even with ordinary sewage it has been observed that the larger forms of plankton (*Paramecium*, for instance) may fail to grow in highly diluted mixtures, although they will grow luxuriantly in higher concentrations.

In the absence of plankton, the bacterial population reaches a position of equilibrium in about 48 hours, beyond which time the absorption of oxygen practically ceases. As the reaction is apparently completed in a relatively short time, the rates of deoxygenation calculated from such data may appear erroneously high.

Whenever necessary, the seeding may be accomplished by adding stale sewage (1 ml. or less per liter) or river water (10 to 20 ml. per liter) to the dilution water. Fresh sewage may be used for inocula-

tion; but as it contains fewer organisms than stale sewage, its use necessitates the addition of larger amounts of extraneous organic material. River water (or water drawn from the receiving body of water) is probably to be preferred.

In the case of chlorinated sewage or sewage effluents, the seeding may not be effective unless all of the chlorine and chloramine compounds have been dissipated. With such samples it is desirable to insure the presence of nitrite- and nitrate-forming organisms.

3. SUPERSATURATION

Samples collected during the winter months or from localities where algæ are actively growing may be supersaturated (over 9.17 p. p. m.) with dissolved oxygen with reference to a temperature of 20° C. To allow for fluctuations in temperature, the following procedures should be applied whenever the dissolved oxygen content exceeds, say, 9.0 p. p. m. The procedures are given in order of preference and in each case the duration of the treatment is to be determined empirically.

(*a*) Suction may be applied to a bottle partly filled with the sample after the temperature of the sample has been raised to approximately 20° C. The release of oxygen is greatly accelerated by agitation.

(*b*) The sample may be mildly preheated in a water bath. The excess oxygen is then liberated by agitation and the sample is cooled to 20° C. The temperature of the water bath should not exceed, say, 30° C.

(*c*) Compressed air may be passed through the sample.

The possibility of diluting the sample with deaerated water should also be borne in mind.

The use of devices for entrapping oxygen liberated from supersaturated samples during incubation is not recommended.

4. UNDERSATURATION

Within reasonable limits, undersaturation is not objectionable. Under certain winter conditions, however, river samples may be received which are deficient in dissolved oxygen although they do not possess a high enough oxygen demand to warrant dilution with aerated water. The dilution water itself, if prepared from distilled water, may be greatly undersaturated with dissolved oxygen.

The oxygen content of an undersaturated sample may be raised by agitating it in a partly filled bottle after bringing the temperature of the water to 20° C.

A fully aerated distilled water should be obtained, if the distillate, suitably cooled, is allowed to drip into the receiving vessel.

CHOICE OF CONCENTRATION

With ordinary river water, dilution is seldom necessary. Sewage and other effluents of excellent quality may likewise frequently be incubated without dilution, at least over the usual 5-day period of incubation.

With normal sewage it is necessary to dilute the samples to such an extent that the depletions of oxygen fall within the limits of, say, 2.0 to 8.0 p. p. m., when the initial oxygen content is 9.0 p. p. m. If the depletions are very small (2.0 p. p. m. or less) the percentage error becomes great. Conversely, it is desirable that 1 p. p. m. of dissolved oxygen or thereabouts be present at the completion of a 5 or 10 day test. For average American sewage, trial concentrations of 2.5 to 5 per cent are suggested.

In work with effluents of good quality or with sewage incubated for 10 or more days at 20° C., it is necessary to insure the presence of relatively large amounts of dissolved oxygen at the close of a test, recent experiments indicating that nitrite formation may be inhibited when the dissolved oxygen content falls below 2 p. p. m. In part, this observation may explain discrepant results obtained at times, over long periods of incubation, with different dilutions of raw sewage. A similar effect may be observed over short periods of incubation when diluted and undiluted effluents are compared, the diluted samples giving much higher results.

With industrial wastes of uncertain or unknown characteristics, several concentrations of sample should be prepared, each 50 per cent of the next highest until the desired range has been compassed. The actual number of concentrations to be incubated will depend on the certainty with which the behavior of the sample is known and it will be governed to some extent by the period of incubation.

DILUTION TECHNIQUE

Starting with a waste which must be diluted prior to incubation, the essential requirement in the preparation of a series of concentrations is that the subsamples be uniform in regard both to distribution of sample and to dissolved oxygen content. Although the technique actually used will depend on individual preferences, the following general considerations should apply:

(*a*) If only one concentration of a waste is required for determining the oxygen demand of a waste over a single period of incubation, it is convenient to prepare a stock solution which may afterwards be transferred to the incubation bottles.

Thus, to prepare a 10 per cent concentration, a liter flask is partly filled with diluting water, 100 ml. of sample are added, and the liter

flask is made up to the mark with dilution water, moving the siphon up and down to secure good mixing. The contents of the flask are then siphoned to three incubation bottles of approximately 300 ml. capacity, the first portion going over being rejected in order to clean the siphon and to clear it of air bubbles. In siphoning the samples to the incubation bottles, the delivery tube should be kept below the surface of the liquid to prevent the mechanical entrainment of air bubbles. As the oxygen content of the dilution water is not greatly changed when this technique is followed, it may be possible to derive the initial oxygen content by computation. The general arrangement is shown in Figure 5.

Alternatively, the sample may be added to a liter graduate, mixing being secured by a suitable stirring device.

More conveniently, the 10 per cent concentration may be secured by adding 100 ml. of sample to a large bottle, followed by the addition of a measured volume of 900 ml. of dilution water or by making up with dilution water to a predetermined mark, depending on the accuracy desired. The sample is then mixed by agitation and siphoned into bottles as before, after air bubbles have disappeared. A large separatory funnel may be used to advantage instead of a bottle. This procedure insures complete mixing, but it presupposes that the initial oxygen content of the sample is to be determined by actual analysis and not by computation.

(*b*) If a series of concentrations of the same waste are being prepared, it may be convenient to put up one concentration stronger than actually desired and to prepare the others from it. Thus, if the desired concentrations are to contain 1.0, 0.5, 0.25, and 0.125 per cent of sample, a stock solution containing 5 per cent of the sample may first be prepared by diluting 25 ml. of the sample to 500 ml. Proper mixing of the stock solution may be secured by moving the siphon up and down while filling or by blowing back the first 50 or 100 ml. pipetted. The required concentrations may then be obtained by taking portions of 200, 100, 50, and 25 ml. from the stock solution and diluting each amount to one liter as described above. This procedure lessens the sampling error, especially if the concentrations are small.

(*c*) Various simplified procedures may be employed in routine work. Thus, when it is desired to dilute a sample with an equal volume of water, it may be convenient and sufficiently accurate to calibrate the bottles with reference to their middle points and to fill them directly with the samples up to these predetermined marks. The bottles are then filled nearly to overflowing with the dilution water.

The dilution technique is greatly simplified when suitable amounts of sample (0.5, 1.0, 2.0 ml., etc.) are added directly to bottles

of known capacity, the bottles then being filled with dilution water. This procedure, however, complicates the computations and it may introduce a considerable sampling error if the amount of sample required is small.

INITIAL DISSOLVED OXYGEN CONTENT

The initial dissolved oxygen content of the dilution water must, of course, be determined in all cases. If the oxygen content of the waste is also known, the initial oxygen content of the diluted wastes may be computed from the amount of waste added. Very frequently, however, it may be difficult or else unnecessary to determine the dissolved oxygen content of the undiluted waste. If the dilution technique is such that the oxygen content of the dilution water is not greatly changed during the manipulations, it may be possible to gain some information regarding the oxygen content of the undiluted waste by a consideration of the value found for the oxygen content of the diluted mixture. The three following cases should be borne in mind:

(*a*) The oxygen content found analytically is the same as that computed on the assumption that the waste was devoid of oxygen; that is, the waste has no dissolved oxygen and no initial oxygen demand. In this case, if the initial oxygen content of the dilution water is 8.0, the dissolved oxygen content of a 10 per cent concentration is obviously 7.2 parts per million.

(*b*) The diluted sample shows a higher dissolved oxygen content than the computed value, i. e., the waste contains some dissolved oxygen.

(*c*) The diluted sample shows a lower dissolved oxygen content than the computed value, i. e., the waste has an immediate oxygen demand. In this case the computed value should be used, as it corresponds to the oxygen which was actually present at the start of the test.

If several concentrations are put up at the same time, the initial oxygen content of one is determined and that of the others is computed. If the concentrations are all less than 1 per cent, the value which corresponds to the oxygen content of the dilution water may be assumed.

Appropriate methods for the determination of dissolved oxygen are given in Part I of this article.

IMMEDIATE OXYGEN DEMAND

In dealing with stale sewage or septic-tank effluents and, also, with certain industrial wastes (for example, sulphite pulp or tannery waste liquors), it is generally observed that the rate of oxygen con-

sumption, during the first few minutes after diluting the sample with aerated water, is appreciably higher than the normal or usual rate of oxygen disappearance. The fraction of the total oxygen demand which is thus satisfied in a relatively short period of time is generally referred to as the "immediate oxygen demand." A similar effect is observed in determining the oxygen demand of sludges.

With fresh sewage, and with iced samples of sewage, the immediate oxygen demand is generally negligible in comparison with the 5 or 10 day oxygen demand, and, if these periods of incubation only are being considered, it may be disregarded. It may, however, form a considerable portion of the 1-day demand, and it can not be disregarded in rate-of-reaction studies or in attempts to estimate the probable effect of a sewage on a receiving body of water at points just below sewer outlets.

General rules can not be given for estimating the immediate or purely chemical demand of industrial wastes, each waste constituting a separate problem. With certain wastes (for example, mine drainage and pickling liquors) the possibility exists of measuring the immediate demand chemically by a direct estimation of known reducing agents (in this instance, ferrous iron). As a rule the pH value of the diluted wastes will exert a material effect on the rate of satisfaction of this purely chemical part of the reaction.

The most satisfactory method of procedure is to follow the disappearance of dissolved oxygen by a gasometric analysis of the air contained in a bottle partly filled with the sample under examination (see reference 3). This procedure permits the determination of the immediate demand of undiluted samples and avoids the experimental difficulties connected with the use of the Winkler method in the presence of much organic matter and other interfering substances.

In the absence of appropriate apparatus, the immediate oxygen demand may be determined, in favorable cases, by preparing a suitable dilution—say, 1:9—of the waste with aerated water of known oxygen content. The bottle is sealed and allowed to stand until, as shown by trial, this relatively rapid reaction is largely satisfied (10 minutes or longer). Its oxygen content is then determined and the immediate demand is computed as in the following example:

	P. p. m.
Initial dissolved oxygen content of the diluting water	8.0
Computed initial oxygen content of a 10 per cent concentration	7.2
Oxygen content found analytically after a given time	6.6
Immediate oxygen demand of a 10 per cent concentration	0.6
Immediate oxygen demand of waste	6.0

SEALING OF SAMPLES

For periods of incubation of about five days at 20° C., the use of seals does not appear essential when a carefully regulated incubator is available, if the incubator can be kept saturated with moisture.

Under less favorable conditions of temperature and humidity control, it may be desirable to provide for volume changes—(*a*) by "jointing" the sample with a rubber collar, as shown in Figure 4; (*b*) by the use of a bottle provided with a large cup (see fig. 5): or, better (*c*) by immersing the samples in water.

PERIOD OF INCUBATION

Relations existing between the oxygen demand values corresponding to various periods of incubation are fully discussed in Public Health Bulletin No. 173. With industrial wastes, the 1-day demand is seldom representative and may be misleading (lag due to seeding or lead due to immediate demand). The 5-day demand, on the whole, appears to bear the most definite relation to the values obtained over other periods of incubation. For that reason it is strongly recommended that this period of incubation be adopted whenever the examinations are restricted to a single interval.

In this connection it is suggested that valuable information may frequently be obtained by conducting a test over several periods of incubation and plotting the data so as to obtain a complete deoxygenation curve.

TEMPERATURE OF INCUBATION

At 20° C. a variation in temperature of one degree causes a change of about 2 per cent in the specific rate of deoxygenation. Consistent results, therefore, will not be obtained unless the temperature of incubation is reasonably constant.

In selecting a temperature of incubation it is to be borne in mind that, in equilibrium with air, water at 37° C. may contain less than 7.0 parts per million of dissolved oxygen, while at 20° C. the saturation value is 9.17 parts per million. Supersaturation and its attendant errors will therefore be encountered when a temperature of 37° C. is adopted. Moreover, the range of oxygen depletion is narrowed considerably when tests are conducted at 37° C. At low incubation temperatures, say, 10° C. or less, consistent results will not be obtained unless the temperature of the samples as collected was also low. In other respects a temperature of incubation of 20° C. strongly recommends itself for general adoption.

When incubators are not available, as in field work, the samples should be stored in the dark as near to 20° C. as possible and a record made of the temperature.

BLANKS

A correction obviously must be applied if the dilution water itself has an appreciable oxygen demand.

If stored tap water has been used for dilution, it may be assumed that it still has a measurable demand even after storage. To determine its magnitude, bottles of the same size as those used for the dilutions are filled with the dilution water, rinsing the siphon before use to avoid contamination. These blanks are incubated along with the diluted subsamples.

If a synthetic dilution water consisting of distilled water with added mineral salts is used, its oxygen demand may be so small as to be negligible in ordinary work. In any event, when the 5-day oxygen demand of a dilution water, synthetic or otherwise, is very small (0.5 p. p. m. or less), it is advisable to determine its magnitude by averaging a large number of 5-day determinations rather than to place complete reliance on any single determination. The average result obtained may then be used as a constant correction factor.

If more than one period of incubation is employed, the oxygen demand of the dilution water over the shorter periods of incubation may approach the experimental error. In such cases negative values may be obtained. Again the correction should be based on the more reliable results obtained over the longer periods of incubation, using conversion factors (*cf.* Public Health Bulletin No. 173). Thus, for distilled water with an average 10-day demand of 0.18 p. p. m., the correction applicable to 1-day results is about 0.04 p. p. m., a negligible figure in most work.

If a correction must be applied, it should be proportioned to the amount of dilution water present. Thus, if the concentration of waste in the diluted subsample is 50 per cent, the correction applied should be one-half of the value found for the dilution water.

If the concentrations are all less than 1 per cent, the correction may be considered equal to the oxygen demand of the dilution water and the same for all concentrations. The method of applying the correction is shown in Figure 6.

Bottles for incubation tests should be cleaned with concentrated sulphuric acid (or cleaning mixture) and thoroughly rinsed with tap water before use. Unless this precaution is taken, the oxygen demand values will be misleading.